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The Determination of α_1-Microglobulin by Means of an Automated Latex Immunoassay

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Summary: Polystyrene (latex) particles coated with human anti-α_1-microglobulin antibodies are used in an automated "kinetic" assay for α_1-microglobulin in urine. For values below 12 mg/l, there was no significant difference between two kinds of standard, but above 12 mg/l the results depend on the origin of the α_1-microglobulin standard. Correlation between values obtained with both standards was good ($r^2 = 0.968$). The method has a between-run imprecision (CV) of 11–16%. Comparison with two commercial enzyme immunoassays gave a bias of −10% to + 40%, while two nephelometric methods differed by 0% to 23%, possibly because the standards used in these methods were of different origin. These results indicate the necessity of standardization of the determination of α_1-microglobulin. The detection limit of our method was 0.8 mg/l, enabling the application of the method for epidemiological investigations.

Introduction

α_1-Microglobulin is a low-molecular mass protein (M_r 31 000), which is also known as protein HC (1–3). In plasma, it forms a complex with IgA, but in urine α_1-microglobulin exists in the free form. α_1-Microglobulin is probably synthesized in the liver and lymphocytes and the protein possibly has an important function in immunoregulation (1–3). α_1-Microglobulin is filtered by the glomerulus, reabsorbed almost completely by the tubulus, and subsequently degraded. The resorption is disturbed with tubular diseases. In several studies, α_1-microglobulin was shown to be a good indicator for tubular disorders of the kidney (3, 4). For toxicologic investigations, it is important to know whether α_1-microglobulin or other tubular proteins such as β_2-microglobulin and retinol-binding protein (5–8) are excreted in enhanced amounts in an early stage of kidney damage.

α_1-Microglobulin has been determined with radial immunodiffusion (9, 10), turbidimetric and nephelometric methods (11, 12, 13), and enzyme immunoassays (14–18). For an assay to be used in epidemiology, it is necessary that proteins and enzymes can be detected in urine of apparently healthy persons. Radial immunodiffusion and turbidimetric or nephelometric methods with detection limits of 3–10 mg/l do not meet this criterion. Enzyme immunoassays (EIA's) have a detection limit low enough for use in epidemiologic research (14–16). Commercial test kits based on an enzyme immunoassay are obtainable from Fujirebio, Japan (no longer available in The Netherlands) and Elias, Germany.

Introduction of polystyrene particles ("latex") led to the development of several sensitive homogeneous immunoassays for low concentrations of various proteins in urine. *Bernard & Lauwerys* (19) published a continuous flow method for β_2-microglobulin, retinol-binding protein (both indicators of tubular proteinuria) and albumin (an indicator of glomerular malfunction), in which antibodies to these proteins were adsorbed to latex and the amount of agglutination was determined by counting residual unagglutinated particles. *Newman* et al. (20) used another kind of polystyrene particles with covalent binding of antibody to the particles.

A standardized method for the manual determination of β_2-microglobulin, retinol-binding protein and albumin, based on coupling of antibodies to latex (LIA), is published by *Herber* et al. (21).

In this study we present an automated method for the determination of α_1-microglobulin, based on the methods as described by *Herber* et al. (21) and *Bernard* et al. (22), that appears to be more sensitive than turbidimetric or nephelometric methods and is cheaper than a commercial enzyme immunoassay. Its analytical sensitivity and precision is sufficient for determination at physiological levels.

Materials and Methods

Materials

- Polystyrene "latex" particles, diameter 0.80 µm, were obtained as a 100 g/l suspension from Rhône-Poulenc, France (Estapor K 080).
- Rabbit monospecific anti-human α_1-microglobulin antibodies were purchased from Dako, Denmark (code A 256, protein concentration 2.9 g/l).
- Bovine serum albumin for the buffers was from Sigma, USA (A 6947) and for preparing the latex-antibody suspension from Calbiochem, USA (fraction V, 126609).
- Glycine, sodium chloride, sodium azide, and sodium hydroxide were from Merck, Darmstadt, Germany.
- Standards for α_1-microglobulin were obtained from Behring (urine concentrate lot 011285, α_1-microglobulin concentration 1160 mg/l) and from Dako, Denmark (α_1-microglobulin calibrator X 938, 535 mg/l).
- Enzyme immunoassay kit, Imzyne α_1-microglobulin, Fujirebio, Japan.
- Enzyme immunoassay kit, Synelisa Alpha-1-Microglobulin, Elias, Germany.
- Urines. Randomly collected urines were obtained from apparently healthy staff members of the laboratory and from patients in a general hospital after consent.
- Controls. Collections of urine from two patients with nephropathy were centrifuged and divided into 2 ml portions and deep frozen. For each run a sample of both urines was thawed and centrifuged again.

Reagents

- GBS stock buffer, pH 9.0, containing 1.0 mol/l glycine, 1.7 mol/l NaCl, and 76 mmol/l NaN_3.
- GBS buffer. GBS stock buffer is diluted 1 + 9 with water.
- GBS-BSA buffer. To 1 liter of GBS buffer 1 g bovine serum albumin (BSA, Sigma A6947) is added and after dissolution the pH is brought to 9.6 with 10 mol/l NaOH. The solution is filtered through a 0.45 µm pore filter (Millipore, USA). This buffer is used for dilution of standard and samples.
- Stabilization buffer. The pH of the GBS stock buffer is brought to 10.1 with 10 mol/l NaOH and the solution filtered through a 0.45 µm pore filter (Millipore, USA).
- Coating solution, containing 0.1 mol/l NaCl and 15 mmol/l NaN_3.

Standards

Comparable to the manual retinol-binding protein determination (24), a series of α_1-microglobulin standard solutions from 1–512 µg/l was prepared.

Instrumentation

An Eppendorf EPOS 5060 automated analyzer (E. Merck, Darmstadt, Germany) was programmed to perform a 6-point "kinetic" assay.

Method

Originally, a method for α_1-microglobulin was chosen resembling the assays for β_2-microglobulin, retinol-binding protein and albumin as described by *Bernard* et al. (23) and modified for a kinetic assay on the EPOS analyzer. For this method, the optimal concentration of anti-α_1-microglobulin antibody to be adsorbed to the latex particles was established. Depending on the lot of polystyrene particles, about 500 µg anti-α_1-microglobulin antibody per 50 mg latex particles produced the best results.

As the final concentration of the latex particles was higher than that for the manual method for retinol-binding protein (24), a wave-length of 405 nm was chosen for the assay on the EPOS analyzer instead of the commonly-used 360 nm.

Preparation of the latex-antibody suspension

Anti-human α_1-microglobulin antibody (150–200 µl, depending on the lot of α_1-microglobulin antibodies) is added to 4 ml freshly prepared GBS buffer. The 10% latex suspension (0.5 ml) is sonicated for 15 minutes at 150 Watt in a waterbath of 20 °C to prevent the temperature of the suspension from rising. The latex suspension is added to the antibody solution and the mixture incubated at room temperature for 60 minutes with constant slow rotation.

The suspension is centrifuged at 27 000 *g* for 10 minutes, the supernatant is decanted and the latex-antibody residue vigorously washed three times with 4 ml coating solution. Finally, the residue is taken up in 10 ml coating solution. This suspension is stable for several months at 4 °C.

For each experiment a dilution of the latex-antibody suspension is prepared as follows:

To 50 mg of bovine albumin (Calbiochem, USA, fraction V, no. 126609) 8.5 ml bidestilled water is added. Latex-antibody suspension (1000 µl) is sonicated for 15 minutes at 150 Watt in a waterbath at 20 °C.

The albumin solution and the latex suspension are mixed and the mixture is sonicated for another 15 minutes. Stabilization buffer (500 µl) is added and the resulting suspension is mixed well.

The reagent container of the EPOS is filled with this suspension. The suspension is stable for three to six hours.

The EPOS variable settings are outlined in table 1. Essentially, 125 µl latex-antibody suspension from the reagent 1 container is mixed with 125 µl[1]) diluted urine, controls or standard and incubated for 598 seconds at 37 °C. The change in absorbance is measured over the course of 897 seconds at 405 nm.

Sample dilutions

Urine samples were diluted 200 and 800 times with GBS-BSA buffer to encompass a large range of α_1-microglobulin values. This

[1]) The minimally required volume in the cuvette of the EPOS is 240 µl. The sample volume/reagent volume ratio is similar to that of the manual method (21).

high dilution was necessary to be able to measure in the steep portion of the calibration curve. The range obtained with a 200 x dilution was 0.8–20 mg/l.

Statistics

For method comparison regression analysis according to *Passing & Bablok* (26) and *Student*'s t-test for paired observations were used.

Results

Calculations

Blank values were subtracted from the absorbance per minute values of samples, controls and standard. The standard curve has a sigmoidal shape (fig. 1). A 4-parameter logit transformation is used to transform the curve into a linear function. The equation as suggested by *Winkler* et al. (25) is used:

$$\text{absorbance/min (transformed)} = \ln\left[\frac{\text{absorbance/min} - a}{b - \text{absorbance/min} - a}\right],$$

where:

a = lower asymptotic value
b = upper asymptotic value

These values are determined by an iteration procedure.

The transformed absorbance/min is a linear function of ln(standard):

$$\text{absorbance/min(transformed)} = c + d \cdot \ln(\text{standard}),$$

where:

c = y-intercept
d = slope of the calibration line.

The resulting calibration line is used to calculate the concentrations of the urine samples. This calculation was performed on-line with data transmission from the EPOS to a personal computer.

Tab. 1 EPOS variable settings.

Unit of result	mg/l
Kinetic mode	yes
Measurement (s)	897
Incubation (s)	598
Preincubation (s)	0
Cycle time	13
6-Point kinetics	yes
Start kinetic	no
Reagent blank	no
Constant	0
Calibration factor	100 000
No. of sample measurements	2
Sample vol (µl)	125
Reagent vol (µl)	125
Temperature (°C)	37
Wavelength (nm)	405

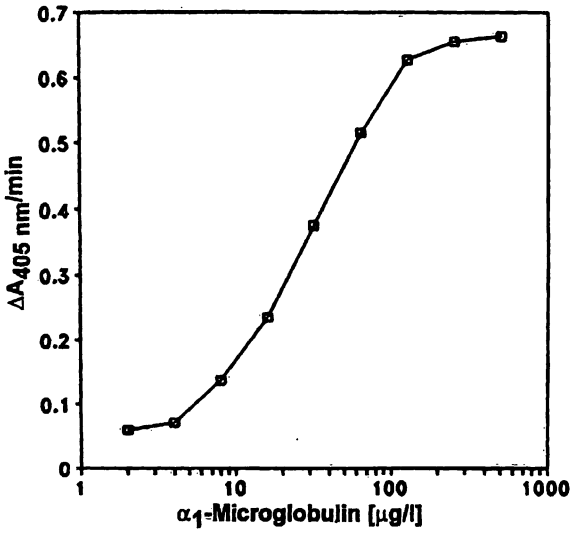

Fig. 1 Calibration curve of α_1-microglobulin.

Detection limit

The lowest standard concentration that gave a delta absorbance/min different from the mean blank + 3 × the standard deviation was 4 µg/l, corresponding to a concentration of 0.8 mg/l in 200-fold diluted urine.

Standards of different origin

During this study two kinds of standard were used, a *urine concentrate* of Behring, Germany, termed "Behring standard" and, after exhaustion of this standard, an α_1-microglobulin standard of DAKO, Denmark, termed "DAKO standard", made from a pathological urine.

Standard curves, ranging from 4 to 512 µg/l, made from both standards were different in shape and position. This caused a difference in values for α_1-microglobulin when the same urines were determined with both standards (fig. 2). For values below 12 mg/l (n = 52, range 1.1–12.0 mg/l), this difference was not significant (95% confidence interval −0.55 to + 0.05 mg/l). Regression analysis gave the following results: DAKO standard = 0.84 Behring standard + 0.8, $r^2 = 0.968$.

When the DAKO standard, suitably diluted, was determined in the enzyme immunoassay with the Synelisa kit, the values fitted very well with the values of the Synelisa standards (fig. 3).

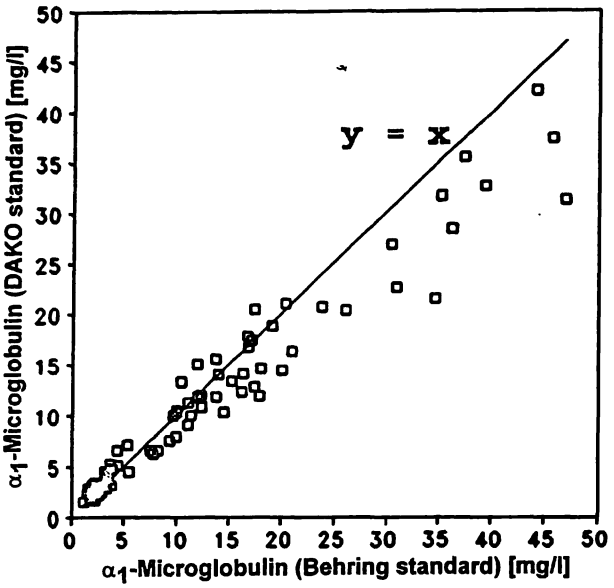

Fig. 2 α_1-Microglobulin concentration (mg/l) of 83 urine samples, determined with the Behring standard (x) and with the DAKO standard (y).

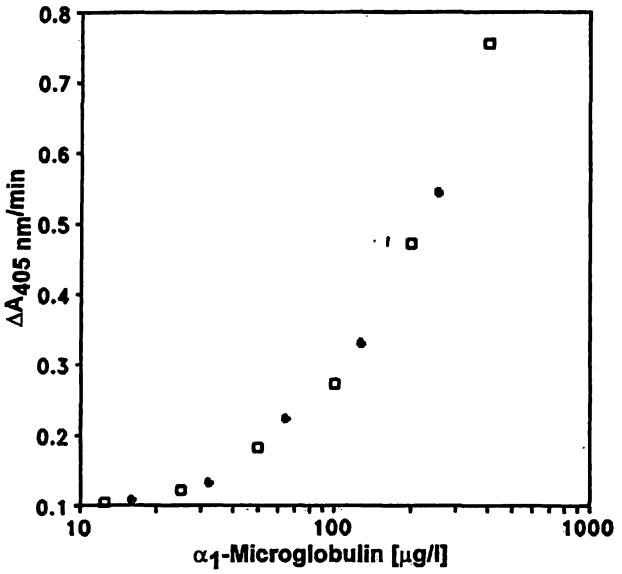

Fig. 3 Absorbance values of different concentrations of Synelisa standard (□) and DAKO standard (+).

Tab. 2 Mean, CV "within-run" and CV "between-run" of the controls.

Control	Standard used in the assay	n	Mean (mg/l)	Within-run CV (%)	Between-run CV (%)
C1	Behring	12	8.1	3.7	11.1
	DAKO	12	8.7	4.6	13.8
C2	Behring	11	5.5	9.1	12.7
	DAKO	11	6.2	9.7	16.0

Controls

In table 2 the mean values of two internal control urines are tabulated, together with the within-run and between-run standard deviation and the between-run relative standard deviation (CV)[2]). The high CV (11−16%) is caused by the inherent imprecision of an immunochemical method as well as by the rather large manual dilution of the urines for the determination.

Recovery

Three different dilutions of the stock standard were added to a urine with a low α_1-microglobulin concentration. At α_1-microglobulin values up to 15 mg/l the mean recovery was 98.2 ± 8.8% (n = 7). At values above 15 mg/l a higher recovery was found (112 ± 1%) (n = 9). The absorbance/minute of samples with an α_1-microglobulin concentration above 20 mg/l was always outside the steep part of the calibration curve in the usual dilution. Consequently, the precision of the determination was much decreased. This emphasizes the necessity of an appropriate dilution of urine samples with an α_1-microglobulin concentration above 20 mg/l.

Stability of the diluted latex-α_1-microglobulin antibody suspension

The assay of a series of urine samples was repeated after two, four and six hours and the values calculated with the calibration curve obtained in the first run. An average increase of 11.5 ± 5.6% was found after six hours. This increase could be caused by an increased rate of spontaneous agglutination of the latex particles. Evaporation from the samples in the tightly capped Eppendorf cups seemed less likely. To avoid deterioration, at least two calibrations per 6-hour period should be carried out.

Comparison with other methods

1. *Nephelometry*

Forty urines, obtained from a general hospital after consent, in which α_1-microglobulin was determined with the LIA, were also determined by an automated nephelometric method at the Public Health Laboratory, Leeuwarden, The Netherlands. Regression analysis revealed a 23% difference in value:

$$LIA = 1.23\ [neph] - 0.6\ mg/l;$$
$$r^2 = 0.981,\ S_{xy} = 1.55.$$

[2]) Unfortunately, the α_1-microglobulin values of both controls differed less than was expected from a preliminary assay.

Urine samples with concentration below the detection limit of 5 mg/l of the nephelometric method were assigned a value of 2.5 mg/l.

2. *Comparison with two enzyme immunoassays*

a. EIA with the Fujirebio kit (see Materials and Method)

Twenty urines with α_1-microglobulin values between 0.7 and 16.8 mg/l were determined with the LIA, (Behring standard) and with the EIA kit of Fujirebio, Japan. The distribution of the values was almost normal.

A good correlation was found between the two methods, but concentrations obtained with the EIA were 40% lower than those determined with the latex method (LIA = 1.64 [EIA] − 0.1 mg/l, $r^2 = 0.983$, $S_{xy} = 0.23$). When the standards included in the EIA kit were run with the LIA, the same bias was found.

b. EIA with the Synelisa kit (see Materials and Method) revealed the same relation between the two methods (LIA = 1.46 [Synelisa] − 1.0 mg/l, $r^2 = 0.986$, $S_{xy} = 0.20$). When the DAKO standard was used in the Synelisa assay, a better relation was found: LIA = 1.08 [Synelisa] + 0.4 mg/l, $r^2 = 0.941$, $S_{xy} = 0.53$.

3. *Comparison of immunoassays with nephelometry (interlaboratory trial)*

Finally, in 17 urines, α_1-microglobulin was determined both with LIA (DAKO standard) and EIA (Synelisa kit). The results were compared with the values obtained by nephelometry in an interlaboratory comparison experiment (Prof. *O. Vesterberg*, National Institute of Occupational Health, Solna, Sweden) (tab. 3). The results of the LIA were not significantly different from those obtained with the Synelisa kit or with the external results.

Tab. 3 α_1-Microglobulin concentrations (mg/l) determined with an external nephelometric method, LIA and EIA (Synelisa).

Urine no.	Nephelometry	LIA	EIA
1	4.5	4.1	4.5
2	3.0	1.3	1.1
3	1.9	6.1	1.8
4	3.9	1.0	1.0
5	6.0	6.0	7.1
6	4.0	2.8	2.8
7	0.05	0.4	0.6
8	0.2	1.3	1.5
9	1.3	1.3	1.0
10	3.0	0.7	0.6
11	1.8	3.5	2.8
12	3.5	2.1	3.05
13	5.0	2.3	2.7
14	14.2	13.0	11.7
15	1.8	2.0	2.8
16	4.7	6.5	3.35
17	1.2	1.1	1.5

Confidence limits:

LIA − nephelometric −0.67 to + 1.20 mg/l;
LIA − EIA −0.40 to + 1.06 mg/l.

Regression analysis:

LIA = 1.0 [neph] − 0.1 mg/l,
$r^2 = 0.840$, $S_{xy} = 0.94$;

LIA = 1.0 [Synelisa] + 0.0 mg/l,
$r^2 = 0.886$, $S_{xy} = 0.75$.

Discussion

Several investigators have compared the results of the determination of α_1-microglobulin, β_2-microglobulin, retinol-binding protein and albumin in urine. *Yu* et al. (5) found a correlation between α_1-microglobulin and β_2-microglobulin ($r = 0.55$, $n = 100$) and between α_1-microglobulin and retinol-binding protein ($r = 0.48$, $n = 100$), but the agreement between β_2-microglobulin and retinol-binding protein was superior for burn injury patients ($r = 0.89$, $n = 100$). As the authors did not give a range of values, the correlation coefficients mentioned above cannot be assessed as to their significance. α_1-Microglobulin followed the same excretion pattern as β_2-microglobulin and retinol-binding protein in the course of a renal disorder, but the magnitude of the changes was smaller.

In our view the determination of α_1-microglobulin in urine as a marker of early tubular damage must be preferred to that of the better known β_2-microglobulin, because the latter is unstable in urine with a pH < 6 and the concentration is much lower than that of α_1-microglobulin.

Hofmann & Guder (27) found that the combination albumin − N-acetyl-β-*D*-glucosaminidase (a tubular enzyme) distinguished better between glomerular and tubular disorders than, for example, α_1-microglobulin − albumin. *Jung* et al. (28), however, found that N-acetyl-β-*D*-glucosaminidase and α_1-microglobulin showed the largest increase in individuals exposed to cadmium compared to controls.

In literature, the intra- and inter-assay precision of the α_1-microglobulin determination with an immunochemical method is given as 3−11% CV (13, 14, 16, 27). This compares reasonably well with the between-run CV of 11−16% of our method with use of the Behring standard. The large manual dilution must have had considerable influence on the results. Use of an automatic dilutor might improve the precision of the assay.

An "in-house" control, prepared from a urine with an increased α_1-microglobulin concentration, can be used to assure the accuracy in every run and between runs, but the possible long-term instability of α_1-microglobulin in urine limits its use.

In our study, the assessment of the accuracy of the latex immunoassay gave variable results, compared to the EIA's and two nephelometric methods. Although calibrators of different origins and the heterogeneity of α_1-microglobulin may be the cause of the discrepancies, this is more likely to occur with the determination of α_1-microglobulin in serum (2, 9) than for the α_1-microglobulin assay in urine, because α_1-microglobulin in serum is partially bound to IgA and assays based on a urine calibrator can give variable results (3). If α_1-microglobulin in urine is to be used as an indicator of an early effect of toxic substances on the kidney, standardization of the assay is necessary to avoid a variety in reference values (29).

The detection level of the LIA (0.8 mg/l) is sufficiently low, compared for example to that of turbidimetric and nephelometric methods. *Itoh & Kawai* (9) reported a measurable range of 0.5–16.8 mg/l with a latex immunoassay. Enzyme immunoassays have better sensitivity (10, 14), but for the Synelisa kit used here, a detection limit of 0.1 mg/l is given by the manufacturer.

The assay of α_1-microglobulin on an automated analyzer can be used for a large series of samples, as can be expected in epidemiological research, while both commercial enzyme immunoassays permitted only a limited number of samples to be determined manually.

Conclusion

α_1-Microglobulin in urine can be determined with a kinetic latex immunoassay on an automated analyzer with a low enough detection limit and sufficient precision for epidemiological research in healthy persons. Standardization of the assay is necessary if the results are to be compared to those of other studies.

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